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Adherence of *Mycobacterium lepraemurium* to Tissue Culture Cells*)

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ABSTRACT

Adherence of *Mycobacterium lepraemurium* to tissue culture cells was examined and compared with that of *M. microti*. *M. lepraemurium* used in the present study was maintained on 1% Ogawa egg yolk medium. This microbe adhered to HEp-2 cells much more than A31 or McCoy cells, though the adherence was unusually low as compared with the large infectious dose. Frequency distribution of the number of bacteria agreed nearly with Polya-Eggenberger distribution. The pretreatment of *M. lepraemurium* with heat or protease increased the adherence of the bacteria to HEp-2 cells, whereas the pretreatment with lipase or hyaluronidase retained the adhering ability. The pretreatment of *M. microti* with heat or protease produced the same effect on the adherence as that of *M. lepraemurium*. These results suggest that adherence of *M. lepraemurium* to HEp-2 cells is prevented by protein-like material on the surface of the bacteria, and that the adherence is independent of specific adhesin-receptor interaction.

INTRODUCTION

Bacterial adherence to tissue cell surface is of importance at the first step of the infectious process. The adherence has a more or less different meaning according to the habitat of microbes. For obligate intracellular parasites, the adherence is followed by their invasion into and multiplication within the host cells without colonization on the surface, whereas colonization generally occurs in common bacteria such as *Escherichia coli* and *Streptococcus*. Although *M. lepraemurium* is not an 'obligate' intracellular parasite because of its capability to grow on artificial culture media, its infectious process is very close to that of obligate intracellular parasites.

In spite of many studies on the adherence of various microbes, no report has been made on that of *M. lepraemurium*. The present paper deals with a preliminary experiment on the adherence of *M. lepraemurium* and *M. microti* to tissue culture cells.

MATERIALS AND METHODS

Bacteria. Since the bacillary preparation of *in vivo* grown *M. lepraemurium* is contaminated by host tissue components and various treatments for taking off the contamination modify the surface structure of the organisms, the bacillary suspension was prepared from *in vitro* grown organisms.

Seven strains of *in vitro* grown *M. lepraemurium* (strains Hawaii, Kurume, Osaka, Kumamoto, Keishicho, Douglas and Odessa) were maintained on 1% Ogawa egg yolk medium. Unless mentioned otherwise, the 78th passaged Hawaiian strain was used throughout the experiments. *M. microti* (TC-48 strain) was maintained on 1% Ogawa whole egg medium.

Cell culture. Established cell lines used were HEp-2 (derived from human epiglottis carcinoma), A31 (a cloned strain of the 3T3 cell derived from a BALB/c mouse embryo) and

*) 加藤雅史，松尾吉恭：鼠癩菌の培養細胞への付着

McCoy cells (derived from human synovial fluid). The former two are epithelial-like cells, and the latter is a fibroblastic cell. They have been routinely cultured in Dulbecco's modified Eagle medium (D-MEM; Nissui Seiyaku Co., Ltd., Japan) supplemented with 10% calf serum (Grand Island Biological Co., U. S. A.), per ml 100 u of penicillin and 100 μg of streptomycin.

Preparation of bacillary suspension. The bacteria grown on the medium for 1 to 3 months were washed twice with Dulbecco's phosphate buffered-saline (PBS) and resuspended in antibiotic-free D-MEM. The bacillary suspension was gently dispersed with a teflon homogenizer and centrifuged at $180 \times g$ for 5 min. The supernatant fluid was decanted and adjusted to O. D. 0. 30 at 540 nm with Coleman Junior spectrophotometer.

Adherence assay. The cells at a concentration of 2×10^4 were introduced into each chamber of four-chambered tissue culture slides (Lab-Tek Products, Miles Laboratories, Inc., U. S. A.) and cultivated overnight. The medium of cultured cells was replaced with the bacillary suspension, and the cells were further incubated statically at 37°C. Three h after incubation, the cells were washed with D-MEM, fixed with methanol and acid-fast stained. The number of bacteria attached was counted tor the total number of 200 cells under a light microscope ($\times 1,000$). The area to read was a center portion on each sample where the distribution of cells was relatively even. Adherence was given as the mean number of bacteria per cell and standard deviation (S. D.).

Pretreatment of bacteria. a) Enzymatic treatments of bacteria were carried out according to the method by Saunders and Miller[5]: PBS-washed bacteria were suspended in each buffered enzyme solution and incubated at 37°C for 60 min. The enzymes utilized are presented in Table 1.

Table 1. Incubation conditions for enzymatic pretreatment of bacteria

Enzyme	Concentration	Incubation buffer
Protease (*B. subtilis*; Sigma, type VIII)	10 U/ml	0.046 M Tris-HCl (pH 7.5) with 0.0115 M $CaCl_2$
Trypsin (Bovine pancreas; Sigma, type III)	10,000 BAEE U/ml[a)]	0.046 M Tris-HCl (pH 7.5) with 0.0115 M $CaCl_2$
Lipase (wheat germ; Sigma, type I)	10 U/ml	0.15 M Na acetate (pH 6.5)
Hyaluronidase (bovine testes; Sigma, type IV)	980 NF U/ml[b)]	0.1 M Na phosphate (pH 6.5) with 0.15 M NaCl

PBS-washed bacteria were suspended in each buffered enzyme solution indicated, and incubated at 37°C for 60 min.

a) N-Benzoyl-L-arginine ethyl ester units.

b) National Formulary units.

b) The heating of bacteria was done in boiling water for 30 min.

After each pretreatment, the bacteria were washed 3 times with PBS and resuspended in D-MEM.

Statistical methods. Statistical analyses were made with the Student' t-test and/or by the Chi-squared test.

RESULTS

Table 2 shows the adherence of the Hawaiian strain of *M. lepraemurium* to HEp-2, A31 and McCoy cells, and Table 3 shows that of *M. microti*. In every instance, the adherence increased as incubation time proceeded. Both *M. lepraemurium* and *M. microti* attached to HEp-2 cells more avidly than the others. Subsequent experiments were performed with HEp-2 cells.

Frequency distribution of number of adherent bacteria in each adherence experiment is shown in Fig. 1. The experimental frequency was very close to Polya-Eggenberger distribution rather than the Poisson one (not presented in the figure). Agreement between the observed frequency distribution and the Polya-Eggenberger one was statistically analyzed by the use of the Chi-squared test. Table 4 shows that the statistical analysis resulted in significant approximation in 5 out of 12 experiments, whereas not significant in 4.

Seven strains of *M. lepraemurium* were tested

Table 2. Adherence of the Hawaiian strain of *M. lepraemurium* to different cell lines

Cell line	Incubation time (h)	Per cent of bacteria-attached cells	No. of adherent bacteria/cell			
			range	median	mean	S.D.
HEp-2	1	24.0	0- 5	0	0.46	0.92
	3	62.5	0-14	2	2.39	2.79
McCoy	1	13.5	0- 3	0	0.21	0.57
	3	19.0	0- 8	0	0.42	1.03
A31	1	2.0	0- 4	0	0.05	0.38
	3	5.0	0- 4	0	0.12	0.52

Table 3. Adherence of *M. microti* to different cell lines

Cell line	Incubation time (h)	Per cent of bacteria-attached cells	No. of adherent bacteria/cell			
			range	median	mean	S.D.
HEp-2	1	75.0	0-12	2	2.22	2.16
	3	97.5	0-29	7	7.97	4.91
McCoy	1	72.5	0- 8	2	1.85	1.75
	3	96.5	0-22	5	5.65	3.85
A31	1	63.0	0-13	1	1.55	1.87
	3	88.0	0-13	3	3.66	2.72

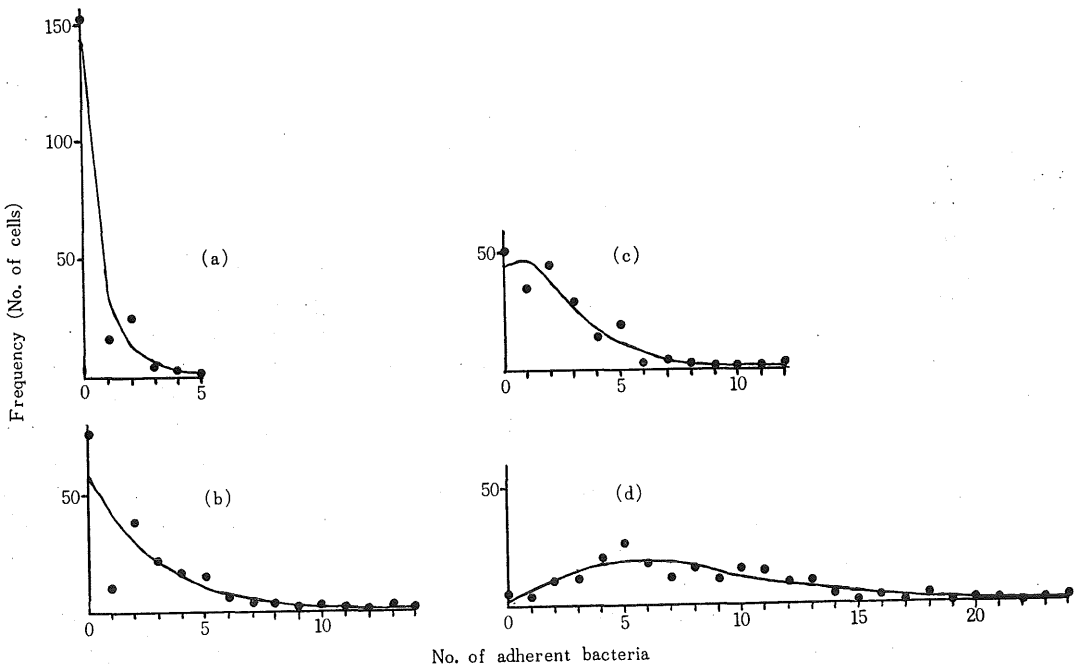

Fig. 1. Frequency distribution of number of adherent bacteria.
Values used were obtained from the experiments shown in Tables 2 and 3: adherence of *M. lepraemurium* to Hep-2 cells at 1h (a) and 3h (b); and that of *M. microti* at 1h (c) and 3h (d). Closed circles indicate observed frequencies. Lines indicate Polya-Eggenberger distribution expected from the mean and variance of experimental values.

Table 4. Agreement between frequency distribution of adherence and Polya-Eggenberger distribution

Bacteria	Cell line	Incubation time (h)	$\chi^{2a)}$	$\chi^2_{0.05}{}^{b)}$	Agreement[c)]
M. lepraemurium	HEp-2	1	24.0181	3.8415	−
		3	35.4194	11.0705	−
	McCoy	1	—	—	N.D.
		3	—	—	N.D.
	A31	1	10.5940	3.8415	−
		3	—	—	N.D.
M. microti	HEp-2	1	15.1081	9.4877	−
		3	14.3140	19.6751	+
	McCoy	1	5.7702	11.0705	+
		3	9.2865	18.3070	+
	A31	1	1.2911	9.4877	+
		3	10.7767	14.0671	+

Experimental values used in this analysis were obtained from the experiments shown in Tables 2 and 3. The approximation to Polya-Eggenberger distribution was evaluated by the Chi-squared test.

a) A observed χ^2 value.

b) A random value of χ^2 ($p=0.05$).

c) +: Observed frequency agrees significantly with the theoretical one ($p=0.05$); −: not significant; N.D.: not determined as a degree of freedom was below zero.

Table 5. Adherence of different strains of *M. lepraemurium* to HEp-2 cells

Strain	Passage	No. of adherent bacteria/cell			
		at 1h		at 3h	
		mean	S.D.	mean	S.D.
Hawaii	78th	4.25	3.47	N.T.[a)]	
Hawaii	5th	0.25	0.60	0.50	0.79
Kurume	20th	2.11	1.82	4.60	3.64
Osaka	46th	0.78	1.48	1.66	2.25
Odessa	46th	0.60	1.20	1.45	1.70
Keishicho	41th	N.T.		3.39	2.69
Kumamoto	45th	1.19	1.62	3.52	2.26
Douglas	41th	1.90	2.15	3.39	2.89

a) Not tested.

on their adherence to HEp-2 cells. As shown in Table 5, the 78th passaged Hawaiian strain attached more to HEp-2 cells than the others.

Next, the effect of various pretreatment of *M. lepraemurium* on adherence was investigated in order to assess what component on the bacterial surface is related to its adherence. The pretreatment of the Hawaiian strain with heat or protease significantly raised the adhering ability while the pretreatment with lipase or hyaluronidase did not (Table 6). The pretreatment with trypsin also caused a statistically significant increase of the adherence, but the ratio of increase was relatively low.

The effect of heat- or protease-pretreatment on adherence was also investigated on *M. microti*. As shown in Table 7, the pretreatment of *M. microti* with heat or protease significantly raised the adherence as was the case with *M. lepraemurium*.

DISCUSSION

Whether an organism observed under a light microscope is attached to or internalized within the host cell is a serious problem in studies on the adherence of intracellular parasites. Recently, Jones et al.[4] clearly distinguished between attached and internalized bacteria according to the method using fluorescent antibody, and revealed that *Salmonella typhimurium* began to enter into HeLa cells after 30 min of incubation, and that the subsequent increase in the number of attached bacteria did not occur whereas the total number of attached and internalized bacteria increased. The number of adherent bacteria shown in our experiments probably represents the sum of attached and internalized bacteria. The results indicate that the adherehce of *M. lepramurium* to tissue

Table 6. Effect of various pretreatment of the Hawaiian strain of *M. lepraemurium* on adherence to HEp-2 cells

	Pretreatment of bacteria[a)]	No. of adherent bacteria/cell		Ratio[b)]	Significance[c)]
		mean	S.D.		
Exp. 1	Control	6.23	4.93	2.01	<0.001
	Heart	12.55	7.50		
Exp. 2	Control	3.40	2.93	1.24	<0.01
	Trypsin	4.23	3.34		
Exp. 3	Control	5.21	4.04	1.82	<0.001
	Protease	9.49	5.48		
Exp. 4	Control	4.02	2.98	1.17	N.S.
	Lipase	4.70	3.84		
Exp. 5	Control	3.70	2.73	0.89	N.S.
	Hyaluronidase	3.30	2.91		

a) See the methods in text.
b) Ratio of pretreated value to control.
c) Since square root transformation of experimental value resulted in transformed variable which was very close to normal distribution, the values derived from its transformation were analyzed by use of the Student' t-test, as compared to control. N.S.: not significant ($p>0.20$).

Table 7. Effect of pretreatment of *M. microti* with heat or protease on adherence to HEp-2 cells

	Pretreatment of bacteria[a)]	No. of adherent bacteria/cell		Ratio[b)]	Significance[c)]
		mean	S.D.		
Exp. 1	Control	8.42	4.84	2.14	<0.001
	Heart	18.05	9.27		
Exp. 2	Control	8.59	4.35	1.60	<0.001
	Protease	13.78	7.75		

a) See the methods in text.
b) Ratio of pretreated value to control.
c) See the footnote in Table 6.

culture cells is mediated by nonspecific interaction rather than specific adhesin-receptor one, because the adherence of *M. lepramurium* was unusually low as compared with a large amount of infectious dose and the pretreatment of the bacilli caused no reduction of the adherence. It may be possibly assumed that *M. lepraemurium* invades the host cells very slowly.

Polya-Eggenberger distribution, one of compound Poisson distributions, has a weakly contagious process as the following: once a very rare phenomenon occurs, it easily spreads around. The adherence of *M. lepraemurium* or *M. microti* in the present assay system remarkably obeys this type of distribution. Experimental values in some cases did not agree significantly with the distribution. These disagreements mainly reflect the difference between the observed and expected frequencies where the number of bacteria is one or two. As *Mycobacteria* easily aggregate in a bacillary suspension, it is difficult to judge whether closely located bacteria on the cell have aggregated or not. Therefore, the preparation and keeping of a single bacterial cell suspension is necessary for reading an accurate number of adherent bacteria.

The pretreatment of *M. lepraemurium* with heat or protease enhanced the adhering ability to HEp-2 cells, whereas other enzymatic treatments retained the ability. These results suggest that the surface of *M. lepraemurium*

contains some protein-like material which interferes with the adherence of bacteria to HEp-2 cells. There has been no report made to describe such an observation so far. The protein-like substance on the bacterial surface is not peculiar to *M. lepraemurium*, since *M. microti* also yielded the same results as *M. lepraemurium* regarding the adhering ability. However, *Actinomyces*, closely related to *Mycobacterium*, is reported to exhibit no increase but remarkable decrease of the adherence with heat or protease pretreatment[5].

The cell wall of *M. lepraemurium* resembles those of other mycobacteria: in that it consists of mucopeptide, arabinogalactan and ester-linked lipid[3]. Although the biochemical approach to the lipid components of mycobacterial cell wall has been made by many researchers, the surface protein has been investigated only with regard to immunological analysis. Caldwell and Buchanan[1] reported that iodination technique using lactoperoxidase method demonstrated two major surface proteins in *M. smegmatis*, one of which was sensitive to pronase. Other authors also stated that an antigenically specific protein destroyed with heat or protease treatment was extracted from *M. leprae*, and suggested that the protein would probably exist on the cell wall surface[2]. These surface proteins are released from the bacteria by Triton X-100 or lithium acetate-EDTA. Then, the relation between the surface protein of *M. lepraemurium* extractable with these mild treatments and the adhering ability will be investigated in future experiments.

Bacterial adherence is known to be affected by hydrophobicity or net charge on the bacterial surface, which has been discussed in detail on the adherence of *Neisseria gonorrhoeae*[6]. In terms of hydrophobicity or net charge, the probability is that the surface protein of *M. lepraemurium* prevents the adherence of the bacteria from HEp-2 cells.

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